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RESEARCH ARTICLE

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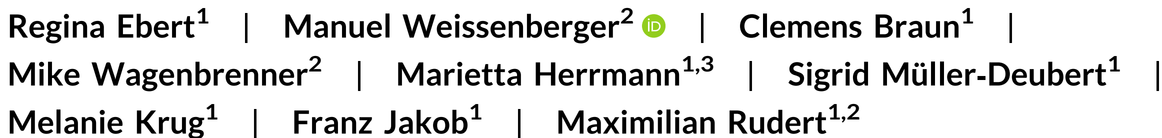
Impaired regenerative capacity and senescence-associated secretory phenotype in mesenchymal stromal cells from samples of patients with aseptic joint arthroplasty loosening



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Abstract

Aseptic loosening of total hip and knee joint replacements is the most common indication for revision surgery after primary hip and knee arthroplasty. Research suggests that exposure and uptake of wear by mesenchymal stromal cells (MSC) and macrophages results in the secretion of proinflammatory cytokines and local osteolysis, but also impaired cell viability and regenerative capacity of MSC. Therefore, this in vitro study compared the regenerative and differentiation capacity of MSC derived from patients undergoing primary total hip arthroplasty (MSCprim) to MSC derived from patients undergoing revision surgery after aseptic loosening of total hip and knee joint implants (MSCrev). Regenerative capacity was examined by measuring the cumulative population doubling (CPD) in addition to the number of passages until cells stopped proliferating. Osteogenesis and adipogenesis in monolayer cultures were assessed using histological stainings. Furthermore, RT-PCR was performed to evaluate the relative expression of osteogenic and adipogenic marker genes as well as the expression of markers for a senescence-associated secretory phenotype (SASP). MSCrev possessed a limited regenerative capacity in comparison to MSCprim. Interestingly, MSCrev also showed an impaired osteogenic and adipogenic differentiation capacity compared to MSCprim and displayed a SASP early after isolation. Whether this is the cause or the consequence of the aseptic loosening of total joint implants remains unclear. Future research should focus on the identification of specific cell markers on MSCprim, which may influence complication rates such as aseptic loosening of total joint arthroplasty to further individualize and optimize total joint arthroplasty.

KEYWORDS

aseptic loosening, mesenchymal stromal cells, regenerative capacity, senescence-associated secretory phenotype

Regina Ebert and Manuel Weissenberger contributed equally to this study.

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1 | INTRODUCTION

About 18% of all women and 10% of all men over the age of sixty suffer from symptoms of osteoarthritis, making it one of the ten most debilitating diseases worldwide.[1] According to a report from the Organisation for Economic Co-operation and Development (OECD), 309 hip replacement surgeries and 223 knee replacement surgeries both per 100,000 population were performed in Germany in 2017.[1] Until 2040, the number of total hip replacement surgeries performed in Germany is projected to rise further and eventually reach 360 procedures per 100,000 residents.[2,3]

Despite—or perhaps because of—the rising number of cases, total hip arthroplasty remains a very safe surgical procedure, leading to a reduction of pain, improved functionality, and therefore high patient satisfaction at 15–20 years follow-up.[4,5] Regardless of these successful outcomes the survival rates of total hip implants are still limited by time. Along with the growing collective of younger patients and the steady increase in life expectancy, this leads to a growing number of revision surgeries.[5,6] Revision surgeries after total hip arthroplasty are not only cost-intensive but also linked to higher complication rates due to differences in medical comorbidities, operation time, and surgical complexity.[7,8]

Common indications for revision surgery after total hip arthroplasty are repetitive dislocations of the hip prostheses, infections, or primary mechanical failures due to implant wear, breakage, and septic or aseptic loosening.[9–11] In particular aseptic loosening of total hip arthroplasty, resulting in a loss of connection between the prostheses and bone or bone cement, has emerged as the leading causes for revision surgery.[9,11] Although septic loosening occurs after germs gain access to the prosthesis either through direct transmission during primary surgery or through secondary bacteremia, the exact pathomechanisms behind aseptic loosening of hip joint replacements are less well understood.[12] Research suggests that wear and debris of implants or bone cement may lead to chronic, low-grade inflammation.[4,13] Interestingly, mesenchymal stromal cells (MSC), which are viewed as a promising alternative for the cell-based treatment of osteoarthritis, maybe a key mediator in the process of aseptic loosening of joint replacements.[14,15] Multipotent MSC carry a characteristic set of surface markers, grow plastic adherent, can be differentiated towards the osteogenic, adipogenic, and chondrogenic lineage in vitro and have been shown to reside in different tissues in and around the arthritic hip joint.[16,17]

The uptake of wear and debris by MSC and macrophages may lead to the secretion of chemokines and proinflammatory cytokines such as interleukin-1ß (IL-1ß) or interleukin-6 (IL-6).[4,18,19] As a result osteoclasts are formed, and further macrophages are recruited leading to bone resorption, osteolysis, and eventually the loosening of implanted materials.[4,12,14] In addition, the uptake of these particles may directly impair osteoblasts function and influence the viability and osteogenic differentiation capacity of MSC.[14] Therefore, functionally deficient MSC could not only increase the risk of aseptic loosening of implanted prostheses but also influence the outcome of future revision surgeries.[15]

Proinflammatory cytokines may also be the cause or the consequence of a senescence-associated secretory phenotype (SASP) in MSC.[20,21] The secretion of cytokines and growth factors can "infect" neighboring cells, leading to impaired regenerative potential and differentiation capacity. The SASP phenotype itself triggers the development of replicative senescence, characterized by permanent cell cycle arrest.

In this current in vitro study, we compared the regenerative capacity of MSC derived from patients undergoing primary hip arthroplasty (MSCprim) due to osteoarthritis to MSC derived from patients undergoing replacement revision surgery after aseptic loosening of total hip and knee joint arthroplasty (MSCrev). The regenerative capacity of both cell types was evaluated by assessing the expression of marker genes for a senescent-associated secretory phenotype as well as the adipogenic and osteogenic differentiation capacity of isolated cells.

2 | METHODS

2.1 | Cell culture of MSCprim and MSCrev

MSCprim and MSCrev were obtained from bone marrow according to the described protocol after total primary hip arthroplasty due to osteoarthritis (MSCprim) and total hip and knee replacement revision surgery (MSCrev).[21] Altogether sixteen patients, seven MSCprim donors (mean age, 61.7 years), and nine MSCrev donors (mean age, 67.9 years), were used. Although all donors for MSCprim underwent primary total hip arthroplasty, four of the donors for MSCrev underwent revision surgery due to aseptic loosening of total knee replacements while five patients suffered from aseptic loosening of implants after total hip arthroplasty. The exact patient demographics and relevant comorbidities are listed in Table 1 for MSCprim and in Table 2 for MSCrev. All knee prosthesis were ultrahigh-molecular-weight polyethylene (UHMWPE)-on-metal implants while all hip prosthesis were UHMWPE-on-ceramic implants. All MSCrev-donors underwent revision surgery due to aseptic loosening of the knee or hip implants. Aseptic loosening of joint implants was diagnosed by clinical examination, radiographic lucent zones along the bone-metal interface and increased uptake in bone scintigraphy. In addition, joint punctures were performed to exclude possible joint infections. MSCprim was isolated from reamings of the femur and hip acetabulum during primary total hip arthroplasty. MSCrev were isolated from bone marrow close to anticipated radiographic loosening zones of hip and knee implants during revision surgery. The procedure was approved by the local Ethics Committee of the University of Würzburg (186/18). Briefly, bone marrow preparations were washed with Dulbecco's modified Eagle's medium, (DMEM/F12) (Life Technologies GmbH) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Bio&Sell GmbH) 1 U/ml penicillin, 100 µg/ml streptomycin (all Life Technologies GmbH), and 50 µg/ml ascorbate (Sigma-Aldrich GmbH) and centrifuged at 250 *g* for 5 min.[22] The so formed pellet was reconstituted in medium and

TABLE 1 Patient demographics of patients undergoing primary total hip arthroplasty for the isolation of MSCprim

Patient demographics MSCprim					
Patient number	Gender (male/female)	Age (years)	Surgical area (hip/knee)	BMI (kg/m²)	Comorbidities
1	Female	64	Hip	29.30	-
2	Male	47	Hip	27.78	Epiphysiolysis capitis femoris as a child
3	Female	60	Hip	52.32	Morbidly obese, high blood pressure
4	Male	80	Hip	22.96	Chronic renal failure, peripheral artery disease
5	Male	68	Hip	25.47	Coronary heart disease with coronary bypass surgery
6	Male	45	Hip	31.20	-
7	Male	68	Hip	30.03	-

Note: MSC derived from patients undergoing primary total hip arthroplasty (MSCprim).
Abbreviations: BMI, body mass index; MSC, mesenchymal stromal cell.

TABLE 2 Patient demographics of patients undergoing revision surgery after total hip or knee arthroplasty for the isolation of MSCrev

Patient demographics MSCrev					
Patient number	Gender (male/female)	Age (years)	Surgical area (hip/knee)	BMI (kg/m²)	Comorbidities
1	Female	53	Knee	29.72	-
2	Female	73	Knee	26.99	-
3	Male	87	Hip	31.22	Obese, high blood pressure, peripheral artery disease
4	Female	67	Knee	32.21	Chronic obstructive pulmonary disease, bronchial asthma
5	Female	62	Knee	25.04	-
6	Female	62	Hip	25.24	Osteoporosis
7	Male	71	Hip	25.39	-
8	Female	55	Hip	21.56	-
9	Female	81	Hip	32.05	-

Note: MSC derived from patients undergoing replacement revision surgery after aseptic loosening of total hip and knee arthroplasty (MSCrev).
Abbreviations: BMI, body mass index; MSC, mesenchymal stromal cell.

washed four times, and the supernatants of the washing steps containing the released cells were collected. Cells were centrifuged and cultivated at a density of 3×10^8 cells per 150 cm² culture flask. Adherent cells were washed after 2 days and cultivated until confluence. Cells were grown at 37°C in a humidified atmosphere consisting of 5% CO_2 and 95% air (Table 3).

2.2 | Growth rate analysis

MSC in passage 0 was trypsinized and reseeded in culture media at a density of 5000 cells per cm². These MSC were allowed to grow until they reached 80%–90% confluence and then passaged at the same cell density onwards until they stopped proliferation. As the cell number of MSC could be determined for the first time at P1, the cumulative population doubling (CPD) was first determined for P2. CPDs were calculated as described previously.[23]

2.3 | Adipogenic differentiation

For adipogenic differentiation MSC was seeded at a density of 2.1×10^4 cells per cm², cultivated until confluence, and incubated in an adipogenic differentiation medium consisting of DMEM high

TABLE 3 Primers used for PCR experiments

Primer	Sequence 5'–3'	Base pairs (bp)	Annealing temp. (°C)
ALPL_for	TGGAGCTTCAGAAGCTCAACACCA	483	55
ALPL_rev	ATCTCGTTGTCTGAGTACCAGTCC		
BGLAP_for	ATGAGAGCCCTCACACTCCTC	293	62
BGLAP_for	GCCGTAGAAGCGCCGATAGGC		
COX4I1	Qiagen sequence	Unknown	60
EEF1A1_for	CTGTATTGGATTGCCACACG	368	60
EEF1A1_rev	AGACCGTTCTTCCACCACTG		
FABP4_for	AACCTTAGATGGGGGTGTCC	177	60
FABP4_rev	ATGCGAACTTCAGTCCAGGT		
IL-1b_for	AAACCTCTTCGAGGCACAAG	169	57
IL-1b_rev	GGCCATCAGCTTCAAAGAAC		
IL-6_for	AAAGCAGCAAAGAGGCACTG	108	60
IL-6_rev	TTTTCACCAGGCAAGTCTCC		
IL-8_for	CATACTCCAAACCTTTCCAC	165	60
IL-8_rev	TCAAAAACTTCTCCACAACC		
LPL_for	CCGGTTTATCAACTGGATGG	110	58
LPL_rev	TGGTCAGACTTCCTGCAATG		
p16_for	GGTGCGGGCGCTGCTGGA	209	60
p16_rev	AGCACCACCAGCGTGTCC		
POU5F1_for	TTTTGGTACCCCAGGCTATG	134	60
POU5F1_rev	AGGCACCTCAGTTTGAATGC		
PPARγ2_for	GCTGTTATGGGTGAAACTCTG	352	61
PPARγ2_rev	ATAAGGTGGAGATGCAGGCTC		
SPP1_for	ACCCTTCCAAGTAAGTCCAA	400	58
SPP1_rev	GTGATGTCCTCGTCTGTAGC		
SAA1_for	GCAAAGACCCCAATCACTTC	127	57
SAA1_rev	GTACCCTCTCCCCGCTTTG		
SAA2_for	CGATCAGGCTGCCAATAAAT	124	60
SAA2_rev	GCCTCATAGCCAGGTCTCCT		

Note: The sequences of the primers are shown with the corresponding product sizes and annealing temperatures.

Abbreviations: ALPL, alkaline phosphatase, liver/bone/kidney; BGLAP, bone gamma-carboxyglutamate protein; EEF1A1, eukaryotic translation elongation factor 1 alpha 1; FABP, fatty acid-binding protein; IL-1ß, interleukin-1ß; IL-6, interleukin-6; IL-8, interleukin-8; LPL, lipoprotein lipase; PCR, ploymerase chain reaction; POU5F1, POU class 5 homeobox 1; PPARγ2, proliferator-activated receptor gamma 2; SPP1, secreted phosphoprotein 1; SAA1, serum amyloid A1; SAA2, serum amyloid A2.

glucose, 10% FCS, 1 U/ml penicillin, 100 µg/ml streptomycin, 1 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 µg/ml insulin, and 100 µM indomethacin. After 2 weeks, cells were harvested for RNA isolation and intracellular lipid droplet staining. Cells cultured in an expansion medium served as a negative control. The medium was changed twice a week.

2.4 | Osteogenic differentiation of hMSC

Human MSC were differentiated into the osteoblastic lineage by seeding 1×10^4 cells per cm^2 in 6-well plates for RNA isolation and histochemical staining. After reaching confluence, the medium was replaced by osteogenic medium consisting of DMEM high glucose,

10% FCS, 1 U/ml penicillin, 100 μg/ml streptomycin (all Life Technologies GmbH), 50 μg/ml L-ascorbic acid 2-phosphate, 0.1 μM dexamethasone, and 10 mM β-glycerophosphate (all Sigma Aldrich GmbH). After two weeks cells were harvested for RNA isolation and mineralized matrix staining. Cells cultured in an expansion medium served as a negative control. The medium was changed twice a week.

2.5 | Histochemical staining

For the detection of calcium hydrogen phosphate and hydroxylapatite in the extracellular matrix, MSC were fixed in methanol, stained with alkaline Alizarin Red S (1% w/v) (Chroma-Schmidt GmbH) for 2 min, and air-dried. For the detection of intracellular lipid vesicles, adipogenic monolayer cultures were stained with Oil Red O solution (Merck). Microscopy images were taken at room temperature with an Axioskop 2 MOT microscope with a 10×/0.3 Plan Neofluar objective and an Axiocam MRc camera (all Carl Zeiss Microimaging GmbH). Staining was quantified using the AutMess tool of the Axiovision Software (Carl Zeiss Microimaging GmbH) by analyzing eight randomly selected pictures per preparation. Significances were tested with student's *t* test.

2.6 | RNA isolation and polymerase chain reaction

Total RNA was isolated from MSCprim, MSCrev, and cells after adipogenic and osteogenic differentiation by using the NucleoSpin RNA II Purification Kit (Macherey-Nagel) according to the manufacturer's instructions. For cDNA synthesis 1 μg of total RNA was reverse-transcribed with Oligo(dT)15 primers and MMLV reverse transcriptase (both Promega GmbH) according to the manufacturer's instructions. For RT-PCR, 1 μl of cDNA was used as a template in a volume of 50 μl. Taq DNA polymerase was obtained from Promega GmbH and primers were obtained from biomers GmbH (see Table 1 for primer sequences and PCR conditions). EEF1α1 was used as a housekeeping gene and PCR bands were analyzed by agarose gel electrophoresis and densitometry using ImageJ. For quantitative PCR, the cDNA was diluted 1:10 and qPCR was performed in 20 μl by using 2 μl of cDNA and 10 μl of GoTaq qPCR Master Mix (Promega GmbH) and 0.25 pmol of sequence-specific primers obtained from biomers. net GmbH or Qiagen GmbH (see Table 1 for primer sequences and PCR conditions). Results were calculated with the efficiency-corrected Ct model with COX4I1 as the housekeeping gene.[24] Significance was tested with REST.[25]

2.7 | Statistical analyses

Statistical analyses were performed using two-tailed unpaired t-test and *p* values less than 0.05 were considered significant. Correlations between values and donor age were calculated with Pearson correlations and *p* values less than 0.05 were considered significant. Further details of the number of independent experiments, MSC donors used and selection of the normalization method are given in the figure legends.

3 | RESULTS

3.1 | MSC isolated from bone samples of patients undergoing aseptic revision surgery show decreased growth rates

In the first step, the growth rates of MSCrev were compared to the proliferation capacity of MSCprim. Determination of the CPDs in both groups revealed higher CPDs of MSCprim (white bars) compared to CPDs determined in MSCrev (black bars) in all passages (Figure 1A). These results were also confirmed by comparing the last confluent passage where a higher CPD number was obtained in MSCprim (13.02 ± 3.55) compared to the CPD of MSCrev (8.37 ± 3.03) (Figure 1B). However, statistical analyses revealed no significant differences. Additionally, the days until growth arrest (Figure 1C) were counted and revealed a significant difference between MSCprim compared to MSCrev (83 ± 13 vs. 109 ± 24 days, *$p \le 0.05$, student's *t* test). The number of passages until growth arrest (Figure 1D) were determined. MSCprim reached higher passage numbers compared to MSCrev (9.20 ± 2.14 vs. 7.25 ± 1.30 passages, no significant difference). No significant correlation was observed between all obtained values and donor age (CPDs, *r*, −0.11 to −0.32; n.s.; CPDs last confluent passage, *r*, −0.54, n.s.; days until growth arrest, *r*, −0.48, n.s.; number of passages until growth arrest, r, 0.34, n.s.; Pearson correlation).

The morphologies of MSCprim and MSCrev were monitored during in vitro cultivation and photographs were taken. Cells did not differ in their morphology in the first passage and MSCrev depicted a spindle-like shaped morphology (Figures S1 and S2). Pictures of two representative donors in different passages are shown in Figure S1. In higher passages, the morphology changed and cells appeared more flattened and enlarged.

3.2 | MSCrev display a senescence-associated secretory phenotype early after isolation

Next, we evaluated if the two cell populations differ in their expression of senescence-associated secretory phenotype (SASP) markers. The expression of serum amyloid A 1 (SAA1), serum amyloid A 2 (SAA2), IL-1β, IL-6, and IL-8 were analyzed by qPCR in MSCrev and MSCprim. SAA1 and IL-1β were significantly higher expressed in MSCrev compared to MSCprim (26.7-fold [±4.10] and 69.5-fold [±16.2], respectively) (Figure 2A). The expression of SAA2, IL-6, and IL-8 was also higher in MSCrev compared to MSCprim but without significance due to high donor variability. Additionally, the expression of the senescence marker p16 as well as the stem cell marker POU class 5 homebox 1 (POU5F1) were analyzed in

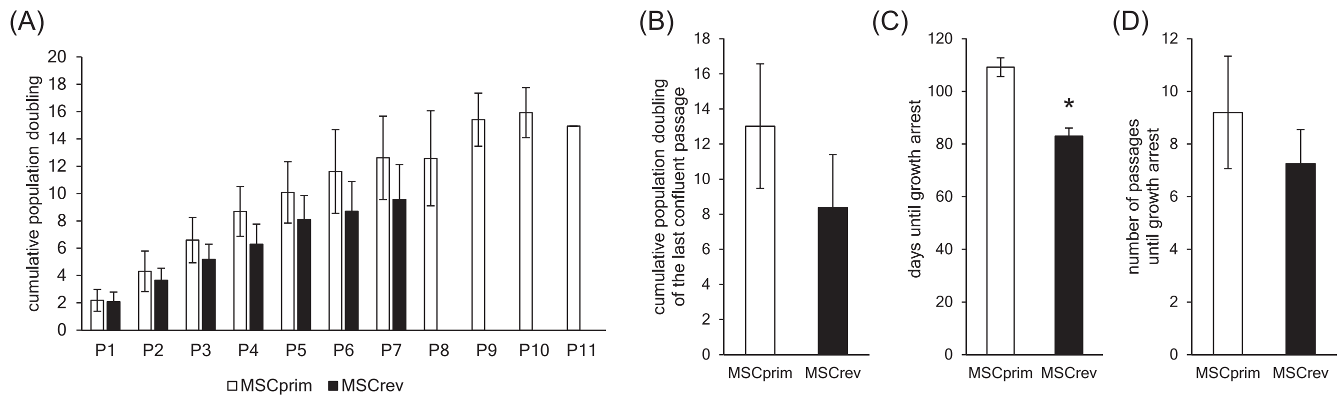

FIGURE 1 MSCrev show impaired proliferation capacity. Cumulative population doubling (CPDs) of MSCrev (black bars) compared to MSCprim (white bars) (A) and determination of CPDs in each passage until growth arrest. Mean CPD in the last confluent passage (B), cultivation days (C) and passage numbers (D) until growth arrest. Results are shown as means ± *SD*; $^*p \le 0.05$, student's *t* test. P1 to P4: MSCprim $n = 5$, MSCrev $n = 4$; P5 MSCprim $n = 5$, MSCrev $n = 3$; P6 and P7: MSCprim $n = 4$, MSCrev $n = 3$; P8: MSCprim $n = 3$; P9 and P10: MSCprim $n = 2$; P11 MSCprim $n = 1$. MSC, mesenchymal stromal cell

MSCprim (white bars) and MSCrev (black bars) (Figure 2B). The stemness marker POU5F1 was expressed in all analyzed cell populations without any difference. MSCrev showed a higher expression of the senescence marker p16 compared to MSCprim (Figure 2B) but this did not reach significance, probably due to high donor variabilities.

3.3 | MSCrev show an impaired in vitro adipogenic differentiation capacity compared to MSCprim

To compare the in vitro differentiation capacity of MSCprim and MSCrev, cells were differentiated towards the adipogenic and osteogenic lineage, respectively. The expression of classical adipogenic markers (fatty acid-binding protein 4 [FABP], lipoprotein lipase [LPL], and peroxisome proliferator-activated receptor-gamma 2 [PPARγ2]) and osteogenic markers (alkaline phosphatase, liver/bone/kidney [ALPL], bone gamma-carboxyglutamate protein [BGLAP], and secreted phosphoprotein 1 [SPP1]) were analyzed by RT-PCR. In addition, lipid droplets were stained with Oil Red O and calcium phosphate crystals were visualized by Alizarin Red S staining.

Visualization of the lipid droplets by Oil Red O (Figure 3A) and subsequent staining quantification revealed an impaired lipid droplet formation in MSCrev compared to MSCprim but no significant difference between the two groups could be observed due to high donor variabilities (Figure 3B). Undifferentiated controls displayed no Oil Red O staining (Figure S2) and therefore could not be quantified. No expression of adipogenic markers could be detected in undifferentiated control samples. Therefore, qPCR quantification was not possible and gene expression was analyzed by RT-PCR

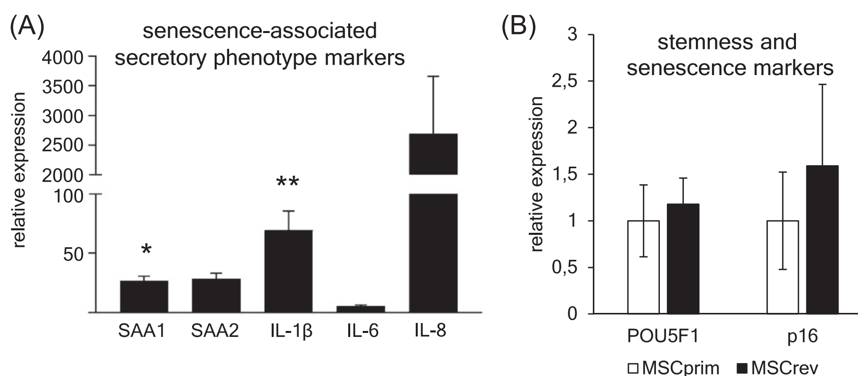

FIGURE 2 Gene expression profile of MSCrev. Relative expression of senescence-associated markers in MSCrev cultivated for one passage (A). Values were calculated with the $\Delta\Delta C_t$ method and were normalized to the expression in MSCprim. COX4I1 (cytochrome c oxidase subunit 4 isoform 1) was used as a housekeeping gene. QPCR data were obtained from technical triplicates out of five independent MSCprim and MSCrev donors and results are shown as mean ± *SD*. Significances were calculated with REST 2009 V2.0.13 software ($^*p \le 0.05$, $^{**}p \le 0.01$).[25] Relative expression of the stemness factor POU5F1 and the senescence marker p16 in MSCprim (white bars) compared to MSCrev (black bars) (B). Results are shown as mean ± *SD*. IL-1ß, interleukin-1ß; IL-6, interleukin-6; IL-8, interleukin-8; POU5F1, POU class 5 homeobox 1; SAA1, serum amyloid A1; SAA2, serum amyloid A2

(Figure 3C), and band intensities were quantified by densitometry. Gene expression intensities of the differentiated samples were normalized to the expression levels of the housekeeping gene EEF1A1. All three adipogenic markers were significantly higher expressed in MSCprim compared to MSCrev after adipogenic differentiation (FABP, 3.5 ± 1.6 vs. 1.2 ± 0.7 $^*p \leq 0.05$; LPL, 3.3 ± 0.9 vs. 2.1 ± 0.6 $^*p \leq 0.05$; PPARγ2, 1.7 ± 0.6 vs. 0.6 ± 0.4 $^{**}p \leq 0.01$; student's *t* test) (Figure 3D).

After osteogenic differentiation and visualization of the mineralized area by Alizarin Red (Figure 4A), subsequent quantification revealed an impaired mineralization rate in MSCrev compared to MSCprim but no significant difference between the two groups could be observed due to high donor variabilities (Figure 4B). Undifferentiated controls displayed no Alizarin Red staining (Figure S2), and therefore could not be quantified. The expression of the osteogenic markers ALPL, BGLAP, and SPP1 were analyzed by RT-PCR (Figure 4C) and densitometrically quantified. All osteogenic markers were higher expressed in MSCprim compared to MSCrev but without significance (Figure 4D).

4 | DISCUSSION

To counteract the rising number of revision surgeries following primary hip or knee joint arthroplasty, recent research has focused on the role of MSC and cellular senescence in aseptic loosening of total joint replacements.[14] Results suggest that phagocytosis of wear debris could lead to the production of proinflammatory cytokines such as IL-1ß, IL-6, IL-8, and TNF-α, which may all contribute to the so-called “particle disease” and support macrophage polarization towards M1 as well as osteoclast differentiation and activity.[14,26] However, these proinflammatory factors are also part of the SASP, which is known to accelerate the senescence-associated proliferation stop in MSC and can improve tissue repair, given that this inflammatory process consists of short-term upregulation and timely inflammation resolution.[27] In contrast, long-term up- or dysregulation of proinflammatory cytokines can have negative impacts on tissue repair and especially bone metabolism.[28] Senescent cells remain metabolically active, do not necessarily undergo programmed cell death but lose most of their functional features.[29] Pajarinen et al.

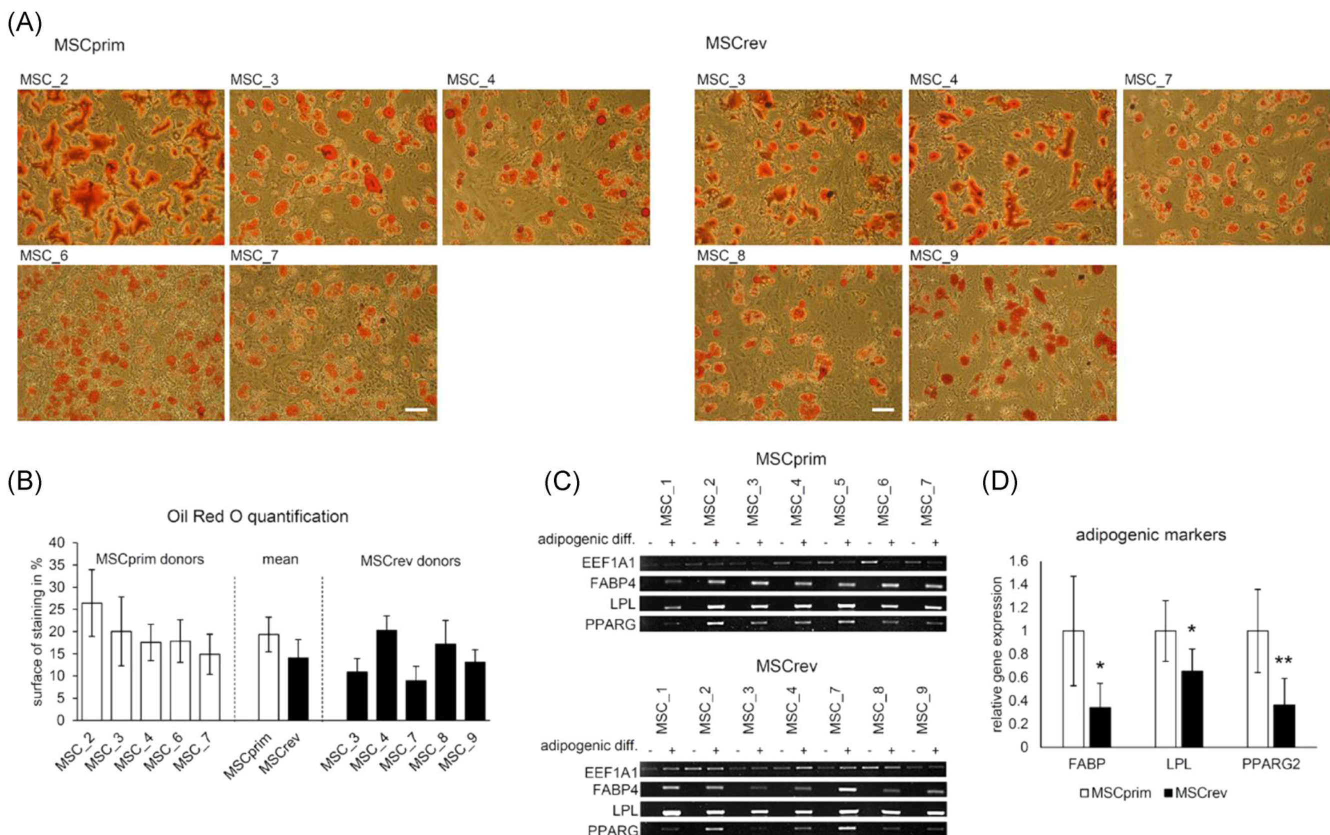

FIGURE 3 Adipogenic differentiation of MSCprim and MSCrev. Lipid droplet staining of MSCprim and MSCrev after two weeks of adipogenic differentiation. Representative images of five donors are shown, bar represents 100 µm (A). For each donor, eight photos were taken and quantified by using the software AxioVision Rel. 4.8 (B). Expression of adipogenic markers in MSCrev compared to MSCprim after two weeks of adipogenic differentiation (C). For quantitation, PCR products were analyzed densitometrically and normalized to the housekeeping gene EEF1A1 (D). Bar graphs display mean transcript levels ±*SD*. Intensities were determined using ImageJ 1.48v software ($^*p \leq 0.05$, $^{**}p \leq 0.01$; student's *t* test). Values obtained from osteogenic differentiated MSCrev were normalized to the levels obtained from MSCprim, which were defined as 1. EEF1A1, eukaryotic translation elongation factor 1 alpha 1; FABP, fatty acid-binding protein; LPL, lipoprotein lipase; PPARγ2, peroxisome proliferator-activated receptor gamma 2 [Color figure can be viewed at wileyonlinelibrary.com]

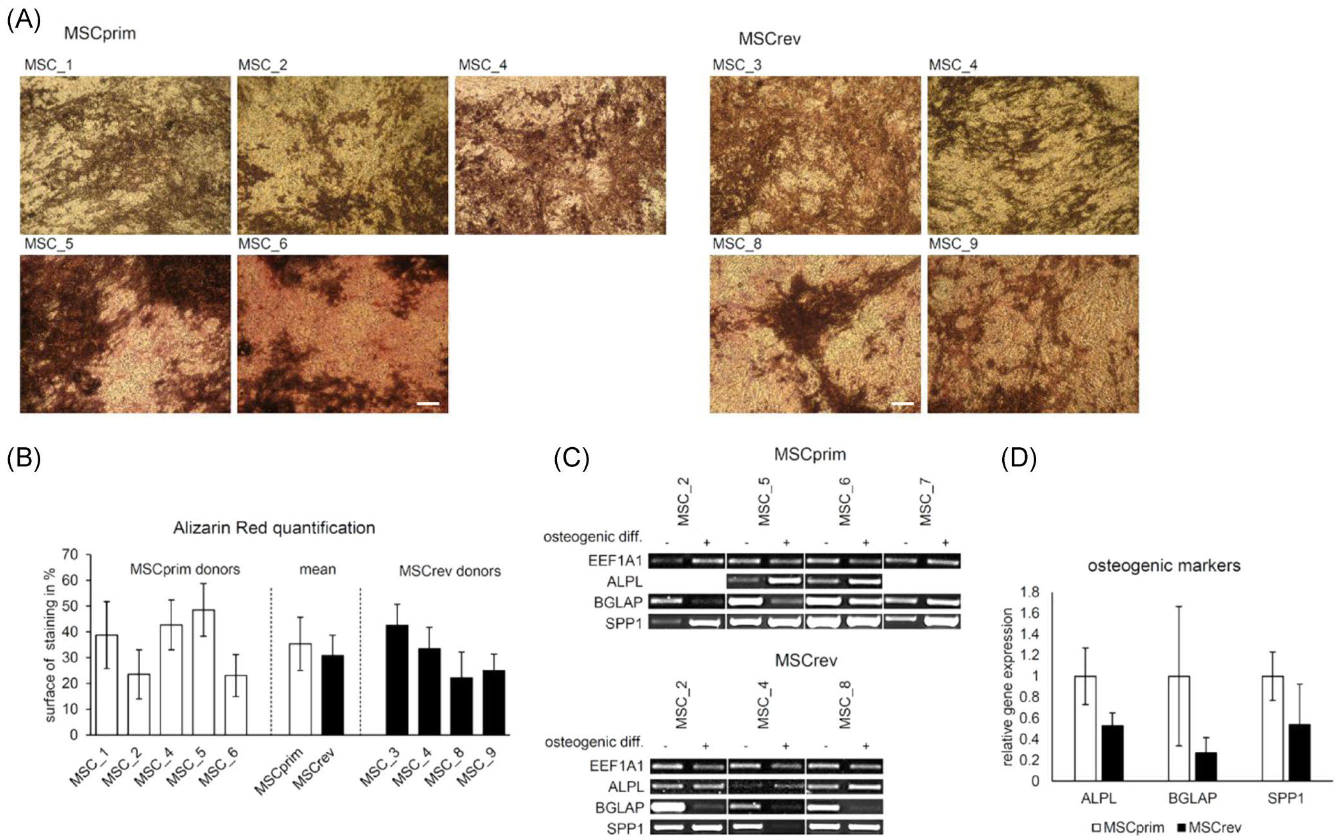

FIGURE 4 Osteogenic differentiation of MSCprim and MSCrev. Alizarin Red staining of the mineralized matrix of MSCprim and MSCrev after two weeks of osteogenic differentiation. Representative images of five (MSCprim) and four (MSCrev) are shown, bar represents 100 µm (A). For each donor, eight photos were taken and quantified by using the software AxioVision Rel. 4.8 (B). Expression of osteogenic markers in MSCrev compared to MSCprim after two weeks of osteogenic differentiation (C). For quantitation, PCR products were analyzed densitometrically and normalized to the housekeeping gene EEF1A1 (D). Bar graphs display mean transcript levels ±*SD*. Intensities were determined using ImageJ 1.48v software. Values obtained from osteogenic differentiated MSCrev were normalized to the levels obtained from MSCprim, which were defined as 1. ALPL, Alkaline phosphatase, liver/bone/kidney; BGLAP, bone gamma-carboxyglutamate protein; EEF1A1, eukaryotic translation elongation factor 1 alpha 1; SSP1, secreted phosphoprotein 1 [Color figure can be viewed at wileyonlinelibrary.com]

discussed that the increased production of IL-1ß, IL-6, and IL-8 following uptake of wear debris may impact functional capacities of MSC as well as increase osteoclast activity.[14] This could result in severe effects on bone metabolism and facilitate aseptic loosening of total joint replacements.[14,21] In addition, negative effects on the proliferation capacity and function of MSC would likely impact the success and outcome of follow-up revision surgeries.[15]

The results of our in vitro study indicate that the proliferation capacity of MSCrev was limited following aseptic loosening of total hip and knee joint arthroplasty, although differences compared to MSCprim did not meet statistical significance. However, MSCrev clearly showed reduced CPD and an earlier growth arrest than MSCprim. In addition, MSCrev expressed higher levels of the proinflammatory and senescence-associated marker genes SAA1, SAA2, IL-1ß, IL-6, and IL-8 in early cell culture passages. The expression of SAA1 and IL-1ß was significantly higher in MSCrev in comparison to MSCprim. Additionally, both MSCrev and MSCprim expressed the stemness marker POU5F1. Regarding their multipotent differentiation capacity, the expression of adipogenic marker genes (FABP, LPL, and PPARγ2) was significantly higher in MSCprim when compared to MSCrev after two weeks of adipogenesis. Although the displayed differences regarding the expression of osteogenic marker genes and Alizarin Red stainings did not meet statistical significance, both investigations showed limited osteogenic differentiation capacity in MSCrev indicating biological relevance.

The tendency towards a lower proliferation capacity together with a very clear early growth arrest in MSCrev presented in our study may be the cause or consequence of aseptic loosening of total hip or knee joint implants. Earlier research by Margulies et al.[15] supports our findings, showing that MSCrev formed 63.9% less cell colonies than MSCprim after cultivation in vitro as shown by fibroblast colony-forming unit (CFUF)-assays. Nonetheless, the donor age provides another important factor potentially influencing proliferation capacity of MSC even though previous research remains inconsistent.[30,31] However, the mean age difference between donors undergoing primary (65 ± 11 years) and revision (70 ± 7 years) surgery in our present study did not meet statistical significance, nor did

we observe significant correlations between all obtained values and donor age.

MSCprim showed signs of a senescence-associated phenotype (SASP) in the first passage with increased expression of proinflammatory and senescence-associated marker genes SAA1, SAA2, IL-1ß, and IL-8 and an increased expression of p16 as an indicator of developing replicative senescence. This observation seems biologically relevant, although some of the values did not reach statistical significance. Our earlier research showed similar results for in vitro aged bone marrow-derived MSC.[21] Interestingly, treatment with recombinant SAA1 self-amplified these effects by binding to the Toll-like receptor 4 (TLR4).[21] Lower relative upregulation of the proinflammatory marker genes IL-1ß, IL-6, and IL-8 in MSCrev during later passages was most likely due to the higher expression levels already during early passages.

As already mentioned, prolonged cultivation of MSCrev and MSCprim in vitro led to an increased expression of p16, which is regarded as a key marker of replicative cellular senescence.[32,33] As we know, this event is more or less a single cell event and such cells get lost during passages in vitro. Interestingly and in plausible consequence, the development of senescence markers did not differ significantly between both cell types and did not impact the expression of the stem cell marker POU5F1, which is considered necessary for maintaining pluripotency in embryonic stem cells (ESCs).[34]

The adipogenic marker genes FABP, LPL, and PPAR γ2 were determined to assess adipogenic differentiation in MSCrev and MSCprim.[35] Previous research by Margulies et al. partly confirmed our findings by showing that the relative expression of the adipogenic marker gene FABP was significantly lower in MSCrev than in MSCprim following adipogenesis in vitro.[15] Although Margulies et al.[15] also observed the formation of significantly fewer adipocytes in MSCrev following adipogenic differentiation, these results could not be reproduced by quantitative evaluation of Oil Red O stainings in our present study.

Similar to the tendencies found in our present study Margulies et al. also showed that the osteogenic differentiation capacity of MSCrev was inferior to that of MSCprim.[15,36] In contrast to our study, these results not only showed a tendency towards limited osteogenic differentiation capacity in revision-MSC but met statistical significance regarding the expression of the osteogenic marker gene BGLAP and the examination of ALP-positive colonies.[15]

One major obstacle regarding our current study is the selection of a representative control group. Although current MSCprim represent cell characteristics of MSCs before primary total hip arthroplasty, the actual goal would be to compare MSCrev to MSCs obtained from donors after primary total hip or knee arthroplasty and without aseptic loosening of implants. This obstacle remains difficult to overcome as the invasive isolation of MSCs located close to the prosthesis can lead to iatrogenic damage of surrounding tissues and normally requires indication for open joint revision surgery. In the current study, we have focused on functional properties of the plastic adherent MSC fraction in vitro, including proliferation, adipogenic and osteogenic differentiation capacity, presenting the most relevant features with respect to the regeneration potential of these cells in vivo. Finally, we cannot exclude the fact that the composition of the adherent cell population or the surface marker expression is likewise altered. It is possible that the conditions after aseptic implant loosening may cause higher contamination of MSC cultures with fibroblasts or macrophages, an aspect that may be addressed in future studies. Nevertheless, the here investigated cell population still reflects the patient's cell composition and functions and thus is of great clinical relevance. Further limitations include the small sample size, which led to high donor variabilities possibly influencing statistical results and significances found in our study. Finally, no expression of adipogenic markers could be detected in control samples. Therefore, we utilized RT-PCR and the expression of adipogenic marker genes in differentiated samples was normalized to the housekeeping gene EEF1A1.

Despite our results, it remains uncertain whether the reduced proliferation and differentiation capacity along with early development of a SASP in MSCrev acts as the cause or consequence of the aseptic loosening of implants after total hip or knee arthroplasty. It is well established that MSC adapt to their local microenvironment, in particular with respect to the inflammatory state of tissue[37] but also mechanical signals.[38] It can be hypothesized that both mechanisms contribute to the altered phenotype of MSCrev seen in our current study. It is very likely that these mechanisms also impact the function of MSC in patients undergoing revision surgeries and consequently implant integration. Future studies will be important to answer these questions, such as the application of wear particles on MSC as well as on MSC/macrophage co-cultures and the analyses of proinflammatory signaling cascades. In any case, however, we provide evidence that not only osteolytic but also impairment of osteoblastic repair capacity may contribute to the progression of implant loosening. Further research including a bigger study population and a more homogenous control group is necessary. Future research should focus on the regenerative capacity of MSCprim during primary joint arthroplasty and determine whether specific cell markers of the regenerative lineages can be used as biomarkers to predict the outcome and may help to develop and to initiate early preventive interventions.

5 | CONCLUSION

MSCrev possessed a limited regenerative capacity in comparison to MSCprim. Interestingly, MSCrev also showed an impaired osteogenic and adipogenic differentiation capacity compared to MSCprim and displayed a SASP early after isolation. Whether this is the cause or the consequence of aseptic loosening of total joint implants remains unclear, but it may contribute to the progression of loosening. The following research should focus on the identification of specific cell markers on MSCprim, which influence later complication rates such as aseptic loosening of total joint arthroplasty to further individualize and optimize total knee and hip joint arthroplasty.

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AUTHOR CONTRIBUTIONS

Research design, interpretation of data, drafting, and approval of the manuscript: Regina Ebert. *Interpretation of data, drafting, and approval of the manuscript*: Manuel Weissenberger. *Acquisition, analysis, and interpretation of data*: Clemens Braun. *Interpretation of data, drafting, and approval of the manuscript*: Mike Wagenbrenner. *Interpretation of data*: Marietta Herrmann. *Analysis of data and approval of the manuscript*: Sigrid Müller-Deubert. *Acquisition and analysis of data*: Melanie Krug. *Research design and interpretation of data*: Franz Jakob. *Research design and interpretation of data*: Rudert Maximilian Rudert. All authors have read and approved the final submitted manuscript and confirm the addition of Dr. Marietta Herrmann as a coauthor.

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SUPPORTING INFORMATION

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